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## Table of Contents

	<u>Page</u>
<b>1. Introduction.....</b>	<b>2</b>
<b>2. Keywords.....</b>	<b>2</b>
<b>3. Overall Project Summary.....</b>	<b>2</b>
<b>4. Key Research Accomplishments.....</b>	<b>5</b>
<b>5. Conclusion.....</b>	<b>5</b>
<b>6. Publications, Abstracts, and Presentations.....</b>	<b>5</b>
<b>7. Inventions, Patents and Licenses.....</b>	<b>5</b>
<b>8. Reportable Outcomes.....</b>	<b>6</b>
<b>9. Other Achievements.....</b>	<b>6</b>

## Introduction

Traumatic Brain Injuries or TBIs are common across both military and civilian populations. In the US, the CDC reports an annual TBI incidence of 1.7 million, with 580,000 TBI-associated deaths in the decade 1997-2007. Worldwide estimates place the number of annual deaths and hospitalizations associated with TBI at 10 million. Low-level laser therapy (LLLT) is unique among the many therapies tested clinically for TBI. Its mechanism of action is biostimulation by near-infrared (NIR) light. In this technical report we have described the progress that has been made on the proposed preclinical and clinical research studies in order to better understand TBI and the potential role of LLLT as a therapy.

## Keywords

Traumatic Brain Injury, TBI, Low level light therapy, LLLT, near-infrared light, NIR

## Overall Project Summary

This work is organized into two objectives. The first objective is a clinical study of LLLT for moderate acute TBI using advanced magnetic resonance imaging as the primary outcome measure. The second objective is a series of preclinical (mouse) investigations to decipher the mechanisms of LLLT such that future clinical studies can incorporate outcome measures that are specifically linked to LLLT's therapeutic mechanism of action. The progress to date for each of these objectives is summarized here.

### Objective 1: Clinical study of LLLT for acute moderate TBI.

Objective 1(i): Acquire and test the optical performance of two LLLT device helmets from Photomedex, Inc.

Completed as reported in the Year 1 annual technical report.

Objective 1(ii): Conduct double-blinded placebo-controlled study of acute LLLT for TBI including collection of neuroimaging, biochemical, and clinical outcome data.

Our initial study design focused on patients admitted to MGH with a Glasgow Coma Score (GCS) between 9-12, an injury within 12 hours, and an anticipated hospital admission of 7 days or longer. This study design was relaxed in year 1 to include GCS 9-12 or GCS 13-15 with abnormal imaging (matching DoD definition of moderate TBI) and an anticipated hospital admission of 3 days or longer. We ran the study with these criteria in year 2 but were unable to enroll subjects.

From Q4 of year 1 through Q3 of year 2, we collected extensive screening logs covering all TBI patients entering the MGH emergency medicine department (ED). In year 2, we used these logs to formulate new inclusion criteria that addresses the enrollment challenges. The central changes

developed in year 2 include:

- (a) Allow recruitment from the MGH ED observation unit. At MGH, most moderate TBIs fall within the GCS13-15 with abnormal imaging category (instead of GCS9-12). These patients are typically admitted to the ED observation unit for 24 hours, and then discharged. Our prior study design did not allow recruitment of these subjects because they were not admitted for the required 3 days. By allowing recruitment from the ED observation unit, the number of eligible subjects increases significantly. To enable this change, we have put in place procedures to allow light therapy to be delivered in the subject's home on days 2 and 3 if the subject cannot return to the hospital (day 1 treatment is performed in the hospital immediately after enrollment).
- (b) Allow recruitment of subjects within 72 hours of injury. In the year 1 study design, we included only patients within 12 hours of injury. Because of the large number of transfer patients at MGH, the time required to perform surrogate consenting, and the uncertainty in defining exact times of injury, this 12 hour window was impractical. The original 12 hour window was motivated by a need to acquire a "baseline" MRI soon enough after the injury that the diffusion parameters would not yet be significantly altered (by the injury). We worked with our neuroimaging team to analyze diffusion imaging results from prior studies. From this analysis, we concluded that imaging can be performed up to 72 hours after injury while still serving as a "baseline" for later imaging timepoints. Thus, we have modified the study to allow recruitment up to 72 hours after injury.

These study design changes were communicated to the DoD in a teleconference with Dr. Crowder and Dr. Williams on June 12, 2015 and were approved by the MGH IRB on 9/29/15 and by the DoD HRPO on 10/07/15.

To implement these study changes, we will require a more extensive collaboration with the MGH ED, and relatively less (but still some) involvement from the MGH Emergency Surgery and Surgical Critical Care department. In year 2, we modified our study team to reflect this shift. Dr. Rajiv Gupta (MD, PhD) from MGH Radiology now serves as the PI of the IRB, replacing Dr. Yeh from MGH Emergency Surgery and Surgical Critical Care. Dr. Gupta is an expert in the field of MRI studies of TBI and has been a critical member of our study team since the start of the award. The amendment to change PI was approved by the MGH IRB on 7/01/15 and the DoD on 7/09/15. In addition to this change in IRB PI, we have built an on-the-ground ED clinical study team including Blair Parry, CCRC, BA (Clinical Research Program Manager, Department of Emergency Medicine) and Dr. Jarone Lee (Department of Emergency Medicine and Department of Surgery). A project manager was also hired through a joint search between our team and the MGH ED and will replace the role previously played by a clinical fellow within the MGH Emergency Surgery and Surgical Critical Care.

Because many of our subjects will be discharged prior to neuroimaging (first imaging procedure occurs on day 2 or 3 after enrollment), we will utilize MRI scanners at the MGH Martinos Center in addition to the MGH clinical scanners. The Martinos Center is a leading neuroimaging research center with state-of-the-art scanners. These scanners have more availability for research scans than the MGH clinical scanners and will aid in scheduling. In year 1, we developed the imaging protocols and physiological monitoring methods for use at the MGH clinical scanners. In year 2, we duplicated these methods to the Martinos scanners. We will still use MGH clinical

scanners for subjects that remain admitted at the time of their imaging procedure.

We are currently training our on-going and new study team members on the revised screening, recruitment and the consent procedures. We plan to begin enrollment under the new study design as soon as Dr. Gupta returns from travel on October 19<sup>th</sup>. We are optimistic that these changes will bring our enrollment rate up to 1-2 subjects per week. However, one challenge we will encounter is patient competition from another ongoing study at MGH (Track TBI). We are working closely with the MGH site leader for Track TBI to identify a plan for patient sharing and are optimistic that enough patients exist in the new inclusion criteria for both studies.

Because of our delays described above and the competition with Track TBI, we anticipate needing to extend this study into year 4. **We therefore plan to file a no-cost extension request to use existing funds to support the study in year 4.** We anticipate that the full study including data analysis will be completed by the end of year 4. Once we have an established enrollment rate, we will be able to better predict this end point.

Objective 1(iii): Perform intermediate and end-study analyses of MRI data to assess: 1) white matter integrity difference between the control and placebo groups using DTI metrics, 2) cerebrovascular reactivity difference between the control and placebo groups, and 3) cerebral perfusion using ASL perfusion maps.

We will perform the analyses once we have collected sufficient clinical data.

Objective 1(iv): Perform an end-study analysis of clinical measures to quantify difference in functional improvement associated with LLLT.

We will perform the analyses once we have completed enrollment.

## **Objective 2: Preclinical investigation of LLLT mechanisms.**

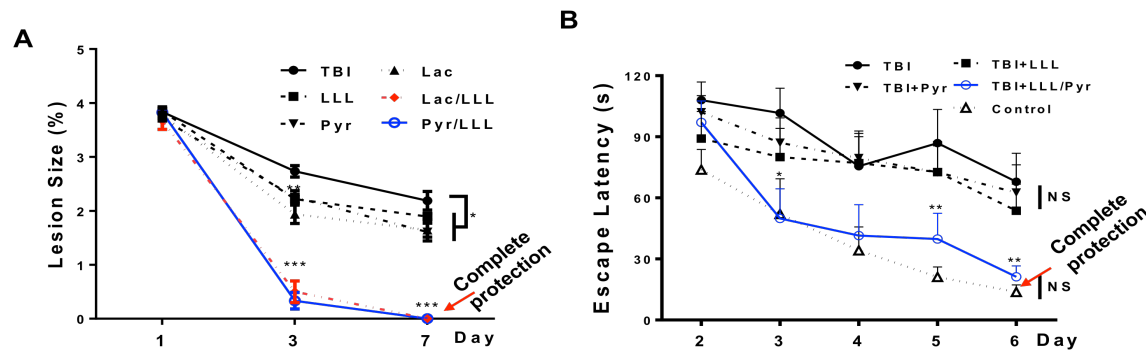
Objective 2(i): Investigate the effect of LLLT on microglial activation in mouse models of TBI.

Microglial activation is usually triggered by cell injury in the injured site. We found that LLLT could prevent or substantially reduce cell death in the injured site due to its ability to preserve mitochondrial functions. Thus, our focus in the past year has been on improvement of low level light therapy (LLLT) effectiveness in treatment of mild or moderate traumatic brain injury (TBI) by enhancing mitochondrial function. Due to vascular damage, TBI leads to hypoxia at the site of injury. Without oxygen, ATP production by the mitochondrial respiratory chain is reduced, and ATP-dependent repair processes are attenuated. In year 1, we showed that high levels of glycolysis, reduced ATP generation, and increased formation of reactive oxygen species (ROS) combine to increase apoptosis in hypoxic neurons. In addition, we showed the striking result that these effects were significantly reversed by LLLT in cell culture, and that this reversal was more pronounced in the presence of either pyruvate or lactate.

In year 2, we demonstrated that mice with TBI that were treated by LLLT and intraperitoneal injection of pyruvate performed normally on memory and learning tests at the end of treatment, while mice with TBI treated with LLLT or pyruvate alone, or untreated control mice displayed either partial or severe deficiency in these cognitive functions (see figure). Consistent with this, we found that the combination of LLLT and pyruvate greatly reduced secondary brain damage in the cortex tissue at the injured site and completely protected the hippocampus tissue from any secondary damage. In contrast, cortical injury progressed into the hippocampus region in the control mice.

The data clearly suggest that energy metabolic modulators such as pyruvate or lactate synergistically enhance the therapeutic effect of LLLT in energy-producing insufficient tissues like injured brain. In addition, these studies suggest metabolic imaging by PET may reveal LLLT target engagement and thereby serve as a powerful outcome measure in future clinical studies of LLLT.

### LLL and Lactate or Pyruvate Additively Protect from Secondary Brain Damage



**A.** TBI causes brain tissue loss, which was slightly prevented by LLL, lactate, or pyruvate alone (2~3% in lesion size over the entire brain), but was completely protected by treatment with LLL and pyruvate or lactate (blue and red lines, 0% lesion size). **B.** The combination treatment fully protected the memory and learning function (Blue) as compared to non-TBI mice (control). The longer escape time means poorer learning and memory.

### Materials and Methods

Mice were subjected to TBI by a standard controlled cortical impact on the left lateral with closed skull and scalp. LLL was performed at 4hr post-TBI using an infrared diode laser of 810nm. Briefly, the mouse was positioned on a plate and covered by aluminum sheet with a 1 cm diameter hole to expose the contusion site on the head. The laser's pulse frequency was 10-Hz, pulse duration 50ms, average irradiance 150mW/cm<sup>2</sup>, a total exposure duration time 4 min, and energy density 36J/cm<sup>2</sup>. Lactate or pyruvate was freshly prepared at a concentration of 50mg/ml prior to scheduled injections and administered intraperitoneally at a dose of 1,000mg/kg at 1 hr post-TBI or 3 hr before the mice were treated with LLL. The mice started Morris Water Maze testing two weeks after TBI for assessment of cognitive deficits. The mice were given one trial session each day for five consecutive days. The time taken to find the hidden platform, also named escape latency (EL), was recorded in each trial. A significant decrease in EL from that of first session was considered as a successful learning. Lesion size was measured in histological sections by Image J and percentages of the lesion size was expressed relatively to the whole brain section.

Objective 2(ii): Image cerebrovascular dysfunction with and without LLLT in chronic mouse models of TBI.

In year 1, we established a new mouse model of TBI that allowed injury through a polyurethane cranial window and optical microscopy of cerebrovascular perfusion immediately after and during recovery from the injury. These methods were described in the first annual report and are repeated below for convenience.

In this second year, we used this model and imaging system to establish the baseline alterations in perfusion after TBI. We observed transient hypoperfusion in the injured hemisphere with minimal if any alterations in the non-injured hemisphere (Figure 1,2). The hypoperfusion lasted for 2-4 hours after injury, and blood at 6 hours appears similar to pre-injury levels. In year 3, we will examine the effect of LLLT on this hypoperfusion. The amendment describing light delivery has been approved by the MGH IACUC and is being submitted to the ACURO concurrent with this report.

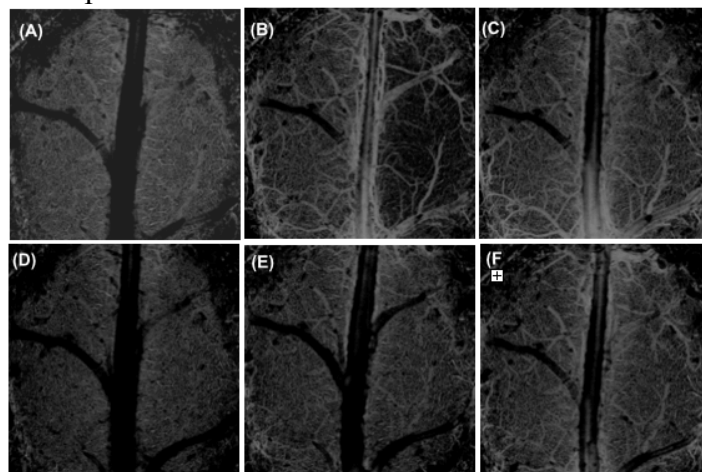


Figure 1. (A). Microphotographs of cerebrovascular capillary network imaged with OFDI through a permanent cranial window in a rodent model. (a) baseline image shows a sagittal view of the detailed capillary network detectable with OFDI in both hemispheres; (b) image taken 15 minutes after mTBI. Injured site (right) shows disruption of vascular network (dark) in comparison with uninjured site (contralateral hemisphere); (c) image taken three hours after mTBI, both hemispheres show vascular flow similar to baseline, including the injured one; (d)(e)(f) images taken at 7 hours, 12 hours, and 24 hours after mTBI. Cerebrovascular flow appears normal in both the injured hemisphere and the contralateral hemisphere.

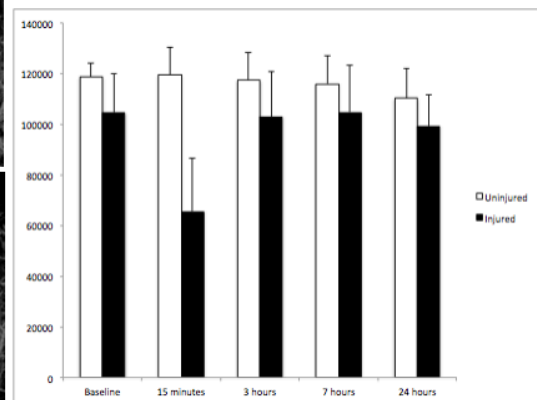


Figure 2. Quantification of active flow as average of pixel area in uninjured (white bars) and injured (black bars) hemispheres at different time points. Results are indicated as average values plus S.E.M.

## **Materials and Methods**

### ***Polyurethane Cranial Window Model***

We have developed a mouse model for chronic cranial window imaging for mild traumatic brain injury (mTBI). Current chronic rodent models rely on a glass coverslip that is removed right before the injury and replaced afterwards. This could potentially confound experimental results due to inflammation and microbleeding. In our chronic mouse model, the glass coverslip has been replaced by a flexible, biocompatible film. The injury is then delivered directly through this material; therefore avoiding unnecessary manipulation of the window prior to the insult.



To perform a cranial window placements, the mouse was anesthetized using Ketamine/Xylazine (90/9mg/kg). Buprenorphine (0.1mg/kg s.c.) was administered thirty minutes prior to surgery. For this procedure the head of the animal is fixed by a stereotaxic apparatus. The skin on top of the frontal and parietal regions of the skull is cleaned with antimicrobial solution and removed in a rectangle-shape from the base of the skull. Using a high speed air-turbine drill with a burr-tip 0.5 mm in diameter, a groove is made on the margin of the drawn circle. This groove is made thinner by cautious and continuous drilling of the groove until the bone flap becomes loose. Cold saline is applied during the drilling process to avoid thermal injury of the cortical regions. The window is sealed with a 7-mm custom-made stainless-steel ring glued with a thin, clear biocompatible polymer film that replaces the traditional coverslip used in standard cranial windows. This is glued to the bone with histocompatible cyanoacrylate. Buprenorphine (0.1 mg/kg s.c.) was administered every 8-12 hours after the first dose and for up to 72 hours after the procedure. The animal is allowed at least 1 week time to stabilize before further procedures are performed.

### ***Controlled cortical impact injury***

We used a controlled cortical injury (CCI) device similar to those previously described in the literature (Flierl et al., *Nature Protocols* 2009) to produce a mild traumatic brain injury, one that disrupts cerebrovascular perfusion but does not fully destroy the pre-injury vascular network. To administer the CCI injury, mice will be anesthetized with inhalant anesthesia. The head of the animal will be fixed by a stereotaxic apparatus. In our model for controlled cortical injury (CCI), the cranial window remains in place, and the injury is performed through a thin film. Buprenorphine (0.1 mg/kg s.c.) was administered 30 minutes before the procedure, then every 8-12 hours after the first dose and for up to 72 hours after the procedure.

### ***Optical frequency domain angiography (OFDA)***

Optical frequency domain angiography (OFDA) is a non-invasive optical imaging modality. Using low-power infrared light, OFDA permits visualization of tissue microstructure, typically at depths of 1.5-2.5 mm and with spatial resolution elements of 2-20 microns, and allows quantitative blood flow measurements in deeper tissues.

Prior to imaging, the mouse was anesthetized using inhalant anesthesia and placed on a polycarbonate stage equipped with a heating pad to maintain body temperature. The duration of each imaging session is approximately 30 minutes. A heating pad was placed under the animal to maintain the animal's temperature at ~37 C during the imaging session. Effectiveness of anesthesia was monitored by respiration rate, toe and tail pinch, and muscular relaxation. After imaging, the animal was placed in the cage and allowed to recover from anesthesia. Perfusion indices were calculated by averaging the angiography pixel values across relevant regions of interest.

### ***Objective 2(iii): Quantify neurogenesis in mouse models of TBI with and without LLLT.***

In year 2, we have shown that mice with TBI caused by controlled cortical impact respond differently according to how often they were treated with transcranial LLLT. We followed these

mice for 4 weeks using the following tests: neurological severity score (neuromuscular coordination), and Morris water maze (spatial learning and memory). A single exposure of 810 nm laser (18 J/cm<sup>2</sup> delivered at 25 mW/cm<sup>2</sup>) delivered to the mouse head at 4 hours after the TBI had a modest beneficial effect up to 4 weeks, while a once a day application for three days (3 exposures) had a much better effect. However a once-a-day exposure for 14 days (14 exposures in total) had no beneficial effects whatsoever, and showed a modest deleterious effect from 7-28 days. In the present reporting period we asked whether this deleterious effect of overdosing was permanent, or if in fact it was only a temporary delay in the manifestation of the beneficial effects of the transcranial LLLT.

We followed these mice for 8 weeks (56 days) following the TBI (Figure 3). We found that indeed the mice that had received an overdose of LLLT treatments (14 exposures) started to improve after week 5, and this improvement continued to increase until day 56. This was observed both with NSS scores and latency in MWM (Figure 4). We sacrificed mice at different time points and examined their brains for markers of neuroinflammation (glial acidic fibrillary protein, GFAP; and ionized calcium-binding adapter molecule 1, IBA1). These markers are typical of activated astrocytes and activated microglial cells respectively. We found that GFAP expression was significantly elevated at 4 weeks which correlated with the abrogation of the beneficial effect of LLLT, and that the elevated GFAP level had gone back to baseline at 8 weeks that correlated with the resumption of the beneficial effects of LLLT.

We conclude that the detrimental effect of 14 daily transcranial LLLT applications to the mouse head, on the recovery from TBI that was produced by three daily laser treatments, is not due to

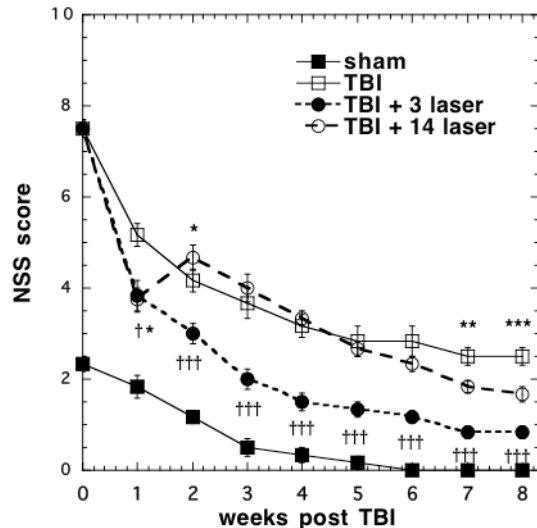


Figure 3. Mean NSS (neuromuscular coordination) scores of mice with moderate TBI receiving different numbers of daily tLLLT applications starting 4 hours post-TBI and followed for 8 weeks. The 14 laser group responds well for first week, but deteriorates at 2nd week, remains non-responsive (same as untreated TBI group) until week 5 and then the beneficial response re-emerges and is significant at weeks 7 and 8.

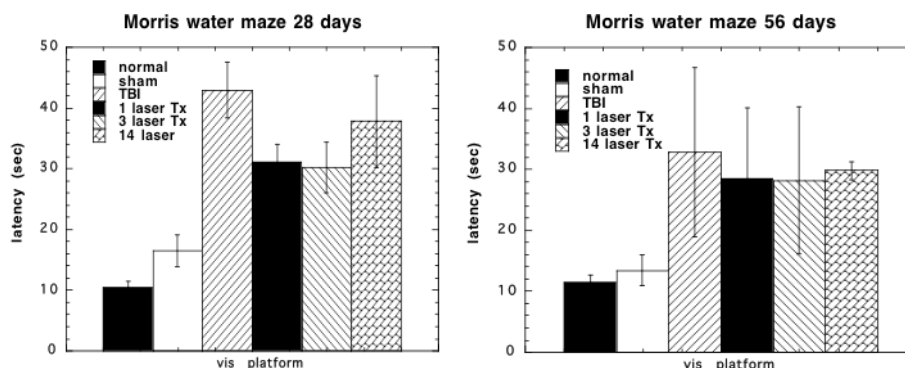


Figure 4. Morris Water maze latency (a measure of spatial memory and learning, lower times mean better memory). The groups of mice received 0, 1, 3 or 14 daily tLLLT applications. At 4 weeks (Fig 2A) the 1 and 3 tLLLT groups showed a significant improvement over untreated TBI, but not the 14 laser group. However at 8 weeks the 14 laser group had “caught up” with the 1 and 3 LLLT groups.

the laser causing permanent damage to the brain. Instead the excessive laser applications only temporarily delay the beneficial effects of the laser. This delay happens because an excessively repeated application of LLLT causes inflammation within the brain. This inflammation delays the beneficial effects of the laser such as BDNF production, neurogenesis and synaptogenesis. When the inflammation subsides the beneficial effects of the LLLT on recovery from TBI resumes.

### ***Rationale for using wild-type in place of transgenic mice***

We originally planned to use strains of transgenic mice. The Dcx-Tk mice which were attempted to be acquired from Dr. Michael Drew's lab at the University of Texas at Austin. The transfer was denied because the vivarium in which they were held was conventional. The option available was to rederive the animals through a commercial vendor such as Jackson, Taconic or CRL. The price of this option at Charles River Labs (CRL) which was recommended by Center for Comparative Medicine was \$8000. The nestin-eGFP-TK mice which were attempted to be acquired from Dr. Steven Kernie's lab at Columbia University in which an MTA was signed. The transfer was denied due to the prevalence of MHV in one of their facilities. The option that was made available was to quarantine the animals at Charles River Labs (CRL). These studies proceeded instead using wild-type mice and relying on histological measures in place of in vivo fluorescence microscopy.

## Key Research Accomplishments

The following were accomplished in the 2nd year of funding:

- The clinical study has been strategically re-designed in order to optimize enrollment using extensive screening logs of MGH TBI patients. These study design changes have been implemented with approval from both the MGH IRB and DoD HRPO.
- We have demonstrated that LLLT reduces functional deficits in mice in part through metabolic pathways, and highlighted enhanced therapeutic responses through the combination of LLLT and metabolic modulators (pyruvate and/or lactate) (*objective 2(i)*). These observations are significant for two reasons. First, it provides new understanding of the mechanism of LLLT and suggests strategies to improve its efficacy (i.e., combination with metabolic modulators). Second, it suggests that metabolic imaging via PET methods may serve as a powerful outcome measure to assess LLLT engagement to the target in future clinical studies.
- We have developed a unique and powerful methodology for measuring vascular alterations after TBI in mice (*objective 2(ii)*). This includes a new surgical model and new intravital microscopies. In years 1 and 2, we demonstrated a pronounced but transient reduction in perfusion at the site of injury. In year 3, we will assess whether LLLT alters this timecourse. This will both identify if LLLT operates through acute vascular mechanisms, and whether perfusion-based imaging should be used to assess LLLT engagement in clinical studies.
- We demonstrated that higher doses of LLLT after injury do not alter the long-term therapeutic benefit of the therapy, but rather delay the healing response (*objective 2(iii)*). This is critical in that it provides guidance on dosimetry in human subject studies.

## Conclusion

The need to improve care for TBI in the civilian and military populations is broadly acknowledged. Acute LLLT for TBI has shown promising results in preclinical studies, and the proposed clinical study is designed to perform necessary pilot studies of acute LLLT in human patients. Specifically, the study will establish safety, demonstrate mechanistic activity, obtain pilot data on effect size for clinical outcome measures, and identify imaging and biochemical biomarkers for use in LLLT optimization and dosimetry studies. In addition, TBI remains a consistent public health challenge in civilian populations. If proven effective, acute LLLT would confer a significant benefit to the broad population by reducing chronic deficits associated with TBI. Success in the acute setting would also suggest follow-up clinical studies of LLLT for the large population of TBI patients with chronic disabilities.

## Publications, Abstracts, and Presentations

### *Presentations at Meetings*

1. Healing with Light. Keynote 1. Optics in Cardiology, Rotterdam, Netherlands. 2015
2. Can all diseases be treated with light? Keynote. The KMALT Annual Conference on Laser Therapy. Dongguk University, Seoul, S Korea. 2015
3. Can transcranial NIR light help the brain to repair itself? Special Seminar for Graduates, Universidade Nove de Julho (Uninove) Sao Paulo, Brazil. 2015
4. Application of Laser Physics in Medicine. Workshop in Mechanisms, Applications, Clinical Training in Photobiomodulation. Laser Research Center, University of Johannesburg, South Africa. 2015
5. Transcranial Laser Effects and Hormesis. Air Force Planning Meeting, Dosimetry and Mechanisms Mediating Responses to tDCS. Amherst, MA., 2015
6. Where We Are Now - Current Status of Research and Technology. 2nd OSA Photobiomodulation Incubator Meeting. Washington DC. 2015
7. Mei X. Wu. Mitochondria, Secondary Brain Damage, and low level light therapy. SPIE, San Francisco, CA USA. Feb. 2015.
8. Tingting Dong and Mei X. Wu. Increased ATP synthesis by LLL and pyruvate/lactate is associated with effective therapy of mild brain injury, presented in Wellman seminar and poster at annual MGH science poster day.

### **Inventions, Patents, and Licenses**

To date we have nothing to report.

### **Reportable Outcomes**

To date we have nothing to report.

### **Other Achievements**

To date we have nothing to report.